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Electrochemical sensors using oligonucleotides as recognition ligands for liquid biopsy in prostate cancer

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ABSTRACT

In men, prostate cancer is the most frequently diagnosed malignancy worldwide. Early diagnosis improves its prognosis, with higher survival rates and a better quality of life for patients. However, diagnosis and characterization of tumors are mainly based on highly invasive tissue biopsies, with an incomplete representation of the entire tumor. In consequence, attention is turning to the so-called liquid biopsies, which provide molecular information about the tumor by detecting its specific components, including proteins, circulating nucleic acids or tumor cells, in bodily fluids such as blood or urine. Recent years have seen the development of a large number of electrochemical sensors employing oligonucleotides for the recognition of prostate tumor components in a minimal invasive way. Here we survey the reported designs, which are categorized as aptasensors and genosensors for the different molecular components identified as prostate cancer biomarkers. We present a critical evaluation of their analytical validity, identifying the challenges of bringing these devices to the clinical practice.

1. Introduction

Precision oncology aims to improve the diagnosis and treatment of cancer by obtaining molecular information about the tumor. Currently it is performed mostly through tissue biopsies, despite being very invasive procedures that can give biased information about the tumor due to its heterogeneity. Tumor cells continuously shed their specific components into body fluids, and consequently the detection of tumor-derived materials in these fluids, commonly referred as 'liquid biopsy', has emerged as a minimally invasive surrogate for tissue biopsy (Bardelli and Pantel, 2017).

In this framework, different forms of liquid biopsy can be exploited not only for diagnosis and prognosis of prostate cancer (PCa) but also for prediction of therapy and disease monitoring, ultimately guiding the best patient care and thus improving their quality of life. Considering that PCa is the most commonly diagnosed male malignancy, the introduction of PCa liquid biopsy assays into the clinical practice would benefit to the about 1 man in 8 that will be diagnosed with PCa during his lifetime. (Culp et al., 2020). However, the integration of PCa liquid biopsy in the clinic requires, as a first step, the development of analytical assays to reliably and accurately measure the analyte of interest with sufficient sensitivity and selectivity (Ignatiadis et al., 2021). The liquid biopsy analytes include proteins and metabolites, circulating tumor cells (CTCs) and extracellular vesicles (EVs), circulating nucleic acids, both tumor-derived circulating DNA (ctDNA) and cell-free RNAs such as microRNAs (miRNAs) and long non-coding RNAs (lncRNAs), which can be found in a range of bodily fluids, mainly blood but also urine or seminal plasma. Biosensors may be quick and cost-effective tools for the assessment of these analytes in clinical settings and as simple devices capable of non-specialist operation at point-of-need.

A large number of transducers are useful in the development of biosensors for PCa biomarkers (Dejous and Krishnan, 2021). Among them, electrochemical ones, which are herein reviewed, constitute a remarkable alternative thanks to their affordable cost, easy handling, and excellent analytical performance. A key element in the construction of the biosensor is the recognition element, and the use of oligonucleotides as probes has inspired increasing research attention due to their wide range of applications (Miranda-Castro et al., 2020). Oligonucleotide-based biosensors are constructed by immobilizing a single-stranded DNA or RNA probe on the sensing phase, which can hybridize with the complementary strand with extremely high efficiency and good specificity. In this way, the detection of the complementary

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strand of DNA or RNA can be easily performed. In addition, it is possible to use functional nucleic acids like aptamers as probes, making possible the detection of other analytes such as proteins, cells or exosomes. In this review, recent advances in biosensors based on oligonucleotides for the detection of established and emerging PCa biomarkers are discussed. The different approaches are categorized at a first level into two classes: aptasensors and genosensors (Fig. 1). Designs in each class are further grouped by the type of molecular marker they detect i.e. proteins, CTCs and exosomes for aptamers, and miRNAs, lncRNAs and ctDNAs for genosensors. We present a critical evaluation of their analytical performance, highlighting their clinical validity. There are excellent reviews explaining the importance of a certain type of biomarker in PCa liquid biopsy, such as cfDNA (Ponti et al., 2021) or exosomal miRNA (Wang et al., 2020). Regarding biosensors technology, the most recent reviews focused on giving an overview of the designs for general cancer biomarkers (Campuzano et al., 2020; Hasan et al., 2021; Koo et al., 2021), while those analyzing PCa applications generally address different types of transducers (Aidoo-Brown et al., 2021; Dejous and Krishnan, 2021) or are restricted to evaluate a particular type of sensor phase design based on the use of nanomaterials (Singh et al., 2019). Reviews on just one biomarker, mainly PSA (Jolly et al., 2015), are usually focused on immunosensors (Traynor et al., 2020). Aptamers for PCa has been recently reviewed for theranostic applications (Campos-Fernández et al., 2021). This work, as far as we know, is the first to review oligonucleotide-based electrochemical sensors as universal platforms for the detection of PCa biomarkers at different molecular levels. Considering the great efforts in PCa biomarkers research, we hope that the tools described in this review will serve as inspiration for the development of new biosensing strategies for alternative and promising PCa biomarkers that will become available. In this way, chemists and biotechnologists will rapidly respond to the demands of clinicians and thus support a better diagnosis, prognosis and clinical management of PCa patients.

2. Aptamer-based assays

2.1. PSA aptamers

Prostate Specific Antigen (PSA) has been the most used biomarker for PCa diagnosis since it was discovered in 1979 (Wang et al., 1979). This 33 kDa glycoprotein is an androgen-regulated serine protease, which is produced by epithelial prostate cells and it is present in both diseased and healthy prostatic tissues. It is released to seminal fluid, where concentration ranges from 0.2 to 3 mg/mL in healthy males. This value decreases in 10^6 times in serum samples because the prostate structure keeps PSA inside, and only a small portion is filtered to the circulatory system. For this reason, the normal concentration in serum is lower than 4 ng/mL (Sharma et al., 2017). In general, PSA concentrations greater than 10 ng/mL in serum are considered an indicative of PCa. However, PSA is not cancer specific. It can be elevated not only in PCa, but also in other benign disorders like prostatitis or benign prostate hyperplasia. For this reason, when values are in the grey zone (2 – 10 ng/mL) the result is not conclusive and it is not possible to distinguish between PCa and other prostatic diseases (Damborska et al., 2017). Thus motivated, extensive research is being carried out to find more specific biomarkers for PCa, mainly proteins as we will discuss later. An incipient trend is the determination of tumoral variants of proteins. In this context, it is important to remark that the capabilities that a tumor cell acquires are related to alterations in the post-translational machinery. Specifically, aberrant glycosylation is a common trait in tumor proteins (Vajaria and Patel, 2017). The detection of the aberrantly glycosylated fraction has been found more specific than the total content of the protein in the case of alpha-fetoprotein (AFP), a biomarker of hepatocellular cancer (Li et al., 2001). Inspired by this successful case, glycoprofiling and detection of glycan structures is an emerging field of study (Díaz-Fernández et al., 2018).

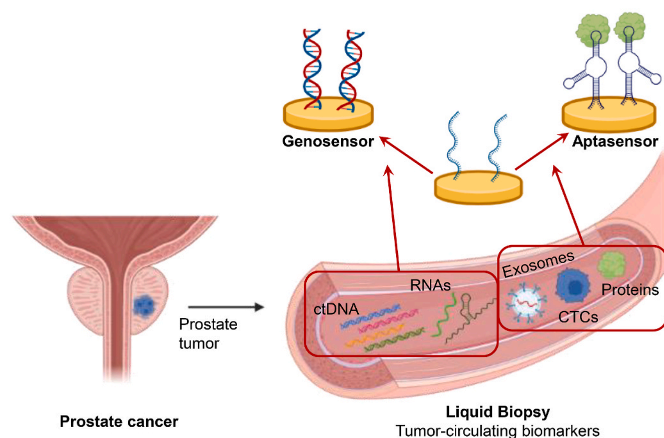

Fig. 1. Schematic illustration of the different PCa related circulating biomarkers and the nucleic acid-based sensors for their detection. Created with BioRender.com.

Biosensors are devices suitable to determine proteins such as PSA due to their sensitive, selective, rapid, reliable and cost-effective ability as diagnostic tool. Their ultimate analytical features strongly depend on the quality of the receptor. Among them, aptamers, which can be obtained in an animal-free, tunable, and easily directed fashion to a specific region of the target, are getting momentum. The resulting aptasensors complement and even can surpass the conventional immunosensors. A key aspect to obtain a robust, reliable aptasensor to be used in complex media is the selection of the appropriate aptamer. For some biomarkers, there are several sequences that claim a specific recognition but concerns about their applicability in real samples is increasing (Álvarez-Martos and Ferapontova, 2017; Zhao et al., 2022; Zong and Liu, 2019). Below we critically discuss the aptamers derived against PSA and other PCa potential targets in the light of the key points one should pay attention to when making the decision of selecting an aptamer for analytical purposes: i) description of the purity and source of the target; ii) the type of library, iii) the use of counter and negative selection steps, iv) complete description of the criteria for sequence classification and selection; v) characterization of the selectivity and the affinity, preferentially by two or more independent techniques.

2010 witnessed the first anti-PSA RNA and DNA aptamers. The DNA one, denoted as ΔPSap4#5, first appeared and was evolved against PSA purified from semen that is expected to be free, not complexed to inhibitors. A 6-FAM labeled library was used and incubated with the PSA spotted on a pre-blocked nitrocellulose membrane. After only three rounds of selection, 11 sequences were obtained. The five with the highest affinity were subjected to three additional rounds of *in-silico* random combination and mutation and then tested for binding to PSA. The aptamer ΔPSap4#5 (5′-TTT TTA ATT AAA GCT CGC CAT CAA ATA GCT TT-3′) showed 48-fold higher affinity for PSA than the round three aptamers. Interestingly, no counter or negative selection steps were performed, but the aptamer showed good selectivity against human serum albumin (HSA), the most abundant protein in serum (Savory et al., 2010). Díaz-Fernández et al. confirmed that the binding site was indeed the peptide region rather than the glycan moiety in the glycoprotein. The aptamer binds the recombinant PSA (70 ± 5 nM) with higher affinity than the natural one (177 ± 5 nM) as it is indicated by the dissociation constants (K_d) estimated by surface plasmon resonance (SPR) with the protein covalently immobilized on the sensor chip (Díaz-Fernández et al., 2019). The preferential derivation of aptamers against the protein region over the carbohydrate moiety is expected because the lack of rigidity of the glycan chain that precludes stable binding. Attempts to improve the Savory's aptamer by *in-silico* evolution combined with molecular simulations and experimental assays with Quartz Crystal Microbalance (QCM) did not show significant results.

Only one aptamer with superior binding was found regardless the starting aptamers, the third round of conventional SELEX (PSAG28, 5′-TTT TTA ATA TCC GGG GAA CGT CGT GGC CCT TT-3′) (Hsieh et al., 2017), or the truncated second *in-silico* generation (5′-TCA GTG AAC TCG CCA TCA AAT ATC-3′) (Lin et al., 2018). Interestingly, the values of K_d estimated by SPR were significantly higher than the reported previously for ΔPSap4#5 (Hsieh et al., 2017), suggesting that steric hindrance is operating when the aptamer is immobilized instead of the protein. Another anti-PSA aptamer (apt2, 5′-GCA ATG GTA CGG TAC TTC CTA TGG CGA TGT GTT GGC TGT GTG TGG GGT GCA AAA GTG CAC GCT ACT TTG CTA A-3′) was obtained without using any counter or negative steps with a very poor description of the process. The methylene-blue-tagged aptamer showed a K_d of 360 ± 80 nM measured electrochemically and a certain degree of affinity for the carcinoembryonic antigen (CEA) and AFP. Interestingly, there are two binding sites for this aptamer in the PSA molecule, which allows the development of an electrochemical enzymatic sandwich assay with modest detectability (0.5 ng/mL) not tested in serum (Li et al., 2018). Finally, in 2016, AS2 DNA aptamer (5′-GGG CGG GGC GGA CGA GAC AGT AAG GGC TGT GGG TGT GGT G-3′) with an impressively high affinity for PSA (K_d = 0.7 nM as measured by SPR) was selected. It is unclear whether PSA is from human semen or the α1-antichymotrypsin complex because both are sold by the company cited. The success of this SELEX procedure in 8 rounds can be related to the efficient partitioning step using acoustophoresis and the simultaneous counter and positive selection. While the PSA is immobilized on magnetic particles (MPs) the three more abundant proteins in serum were added in solution, so the DNA sequences anchored to the MPs are acoustophoretically separated from those free or bound to counter-proteins, without the need of washing steps (Park et al., 2016).

The first RNA aptamer was evolved against a recombinant PSA expressed in *E. coli* with a GST tag. Two parallel SELEX were performed: one with a negative step to remove sequences binding to the GST tag and another one with a counter step to remove sequences binding to the inactive proPSA form. Both SELEX processes converged into two sequences; one of them, named 1.1 (5′- CCG UCA GGU CAC GGC AGC GAA GCU CUA GGC GCG GCC AGU UGC-3′), was selected for characterization because its higher abundance. It showed preferential affinity for the active PSA form but the affinity constant was not estimated (Jeong et al., 2010). Two reasons can explain that this aptamer has not been used in any analytical approach. On one hand, the protein used for the selection and characterization is not glycosylated. On the other hand, the stability of the unmodified RNA is shorter than an hour as it was later demonstrated (Svobodova et al., 2013). These authors solved both problems by performing a new SELEX using an endonuclease resistant 2′F-RNA library and natural PSA from seminal fluid. After 13 rounds of selection with appropriate counter steps at 37 °C, S2 (5′- 2′F-AGC UCC AGA AGA UAA AUU ACA GGU ACG GUU CAC GCC UGU CUC AUG CUG ACU AAG AAA GUU UAG CAA CUA GGA UAC UAU GAC CC-3′) and R3 (5′-2′F-AGC UCC AGA AGA UAA AUU ACA GGU CCA GGC GCG UUA GCA AAA CCG CGG AUC AAA CUU AGU UGA CAA CUA GGA UAC UAU GAC CC-3′) were selected according to their strong secondary structure. Both were selective for PSA against streptavidin and BSA, showing the success of the negative selection step. The one with a modest K_d (630 nM) partially inhibited the PSA activity.

In the search for glycan recognition two aptamers were obtained. Using the recombinant PSA in the counter step, the selection was directed toward the carbohydrate moiety. It resulted in an aptamer PSA-1 (5′-GGA CGG TTG CGC TAT ATT TAA CCA AAA GTC TGG ATT AAC-3') that binds the external sugars without recognizing the peptide (Díaz-Fernández et al., 2019). This feature is analogue to the lectins but with improved affinity. It also confers it versatility because it can be used to detect other glycoproteins with terminal sialic acids in combination with another receptor that binds the peptide region. A step forward is the pioneer development of an aptamer with binary recognition, that is, simultaneous binding to the peptide and the glycan regions (Díaz-Fernández et al., 2020). The successful direction toward the glycan-peptide site was achieved by using a lectin specific of the core fucose present in the innermost sugar as an elution molecule instead of the universal heated water. This ideal receptor, denoted as PSAG-1 (5′-GAG CGG GGT TGC TGG GAT GAT AAG GCC CCT TTG ATG TCT G-3′) was also truncated after computational modeling (T-PSAG-1, 5′- GAG CGG GGT TGC TGG GAT GAT AAG GCC CCT TT-3′) without significant loss in affinity. Both can be used alone for PSA detection.

2.2. PSA aptasensors

Significant efforts have gone into the fabrication of aptamer-based sensors for PSA detection, both optical or electrochemical biosensors (Ghorbani et al., 2019). In this section we discuss the recent advances in those using electrochemical transduction applied to liquid biopsies since 2018, starting from the simplest configurations. The analytical performance of the different approaches is summarized in Table 1.

Direct assay is the preferred format and the most common one because it is based on a change in the analytical signal occurred as a result of the aptamer-protein recognition reaction. A significant hurdle in the development of these sensors, however, is that most transduction technologies do not directly provide a readily detectable output upon the binding receptor-target. Different techniques, such as electrochemical impedance spectroscopy (EIS), cyclic voltammetry (CV), differential pulse voltammetry (DPV) or square wave voltammetry (SWV) have been applied with this aim, using a redox probe in solution, typically the $[Fe(CN)_6]^{3-}/[Fe(CN)_6]^{4-}$system, to monitor the binding (Fig. 2A). These are label-free but not reagent-less strategies. EIS is the technique with the highest number of described assays. Ibau and co-workers have proposed a single-mask gold interdigitated triple-microelectrodes biosensor by using an amino-silanization technique where ΔPSap4#5 aptamer was anchored. They obtained a linear dynamic range from 0.5 ng/mL to 5 μg/mL of PSA in undiluted spiked human serum, with a limit of detection (LOD) of 0.38 ng/mL in buffer and 0.51 ng/mL in serum. The sensor is selective enough to discriminate PSA from other proteins presented in serum samples, like human serum albumin and human glandular kallikrein 2 (7-fold and 8-fold decrease versus 5 ng/mL of PSA, respectively) and stability is also acceptable taking into account that the signal was reliable for 6 days before starting degradation below 10% (Ibau et al., 2019). Regarding shelf life, a glassy carbon electrode (GCE) modified with TiO_2-reduced graphene oxide (rGO) hybrid nanosheets can be used up to 30 days with a minimal analytical signal reduction of 4–7%. No interferences were reported and it covers the clinical threshold value and the grey zone successfully. In addition, spiked serum samples were studied and compared with an immunoradiometric assay (Karimipour et al., 2019). The use of nanomaterials in biosensors is on the rise, and this is a good example of how they can improve analytical characteristics. Another nano-engineered surface was built on GCE using TiO_2 nanoparticles and silk fibroin nanofiber (SF) composite that shows two linear response ranges starting at the fg/mL. Spiked serum samples were analyzed and well compared with a chemiluminescent microparticle immunoassay. LOD is an important analytical characteristic, and this impedimetric aptasensor is able to reach an impressive 0.8 fg/mL (Benvidi et al., 2018). This value leads to consider whether it is really necessary to achieve these low values. The grey zone is the uncertain concentration so, in advance, is not required. However, it is true that once a patient has undergone surgery for PCa, the recurrence monitoring is performed by measuring the variation in PSA concentration over time, at the level of hundreds of pg/mL.

Although the above impedimetric sensors have shown excellent analytical performance, a major drawback remains: the limited clinical specificity of PSA detection. This problem has been addressed by designing a dual sensor platform for the quantification of glycosylated and total PSA using two different aptamers (PSAG-1 and ΔPSap4#5, respectively). Through an empiric index named as glycan score (ratio

Table 1
Characteristics of the reported aptasensors for PSA.

Technique	Type of assay	Aptamer	Linear range	LOD	Sample	Reference
EIS	Direct	PSAG-1 and ΔPSap4#5	glycosylated PSA 0.26–62.5 ng/mL total PSA 0.64–62.5 ng/mL	glycosylated PSA 0.26 ng/mL/ total PSA 0.64 ng/mL	serum	Díaz-Fernández et al. (2021)
EIS	Direct	ΔPSap4#5	0.034–0.057 ng/mL	0.026 ng/mL (buffer)/0.028 ng/mL (serum)	spiked serum	Nxele et al. (2022)
EIS	Direct	ΔPSap4#5	0.1 pg/mL - 100 ng/mL	0.085 pg/mL	serum	Li et al. (2019)
EIS, DPV	Direct	ΔPSap4#5	EIS 1–200 pg/mL DPV 2.5–90 ng/mL	EIS 0.5 pg/mL DPV 1.5 ng/mL	serum	Jalalvand (2019)
EIS	Direct	ΔPSap4#5	0.003–1000 ng/mL	1 pg/mL	spiked serum	Karimipour et al. (2019)
EIS	Direct	ΔPSap4#5	0.5–5000 ng/mL	0.38 ng/mL (buffer)/0.51 ng/mL (serum)	spiked serum	Ibau et al. (2019)
EIS	Direct	ΔPSap4#5	2.5 fg/mL - 25 pg/mL and 25 pg/mL - 25 ng/mL	0.8 fg/mL	spiked serum	Benvidi et al. (2018)
EIS	Direct	ΔPSap4#5	1 pg/mL - 1 ng/mL	0.71 pg/mL	serum	Duan et al. (2018)
DPV	Direct	ΔPSap4#5	1 pg/mL - 200 ng/mL	0.077 pg/mL	serum	Hassani et al. (2020)
DPV	Direct	ΔPSap4#5	1–300 ng/mL	280 pg/mL	spiked urine and serum	Argoubi et al. (2018)
EIS DPV	Direct	ΔPSap4#5	0.14–11.6 ng/mL	0.14 ng/mL (EIS, DPV)	spiked serum	Srivastava et al. (2018)
SWV	Direct	ΔPSap4#5	0.5–350 pg/mL	0.14 pg/mL	spiked serum	Soleimani et al. (2020)
chronocoulometry	Direct	modified ΔPSap4#5	1–500 pg/mL	0.5 pg/mL	spiked serum	Jiang et al. (2021a)
SWV	Direct, reagentless	ΔPSap4#5	10 pg/mL - 500 ng/mL	1.24 pg/mL	spiked serum	Zhao et al. (2021)
DPV	Direct	ΔPSap4#5	50 pg/mL - 50 ng/mL	28 pg/mL	spiked serum	Zhao et al. (2019)
DPV	Direct reagentless	ΔPSap4#5	0.05–200 ng/mL	10 pg/mL	serum	Wei et al. (2018)
DPV	Direct reagentless	ΔPSap4#5	0.025–205 ng/mL	8 pg/mL	spiked serum	Zhang et al. (2018a)
SWV	No-direct self-reported	modified ΔPSap4#5	0.5 pg/mL - 50 ng/mL	0.15 pg/mL	serum	Liu et al. (2018)
DPV	Sandwich	Apt2 and ΔPSap4#5	0.001–10 ng/mL	0.25 pg/mL	whole blood	Wang et al. (2022)
DPV	Sandwich	Antibody and ΔPSap4#5	0.05–100 ng/mL	0.017 ng/mL	spiked serum	Meng et al. (2019)
chronoamperometry	Sandwich	PSA-1 and ΔPSap4#5	0.66–25 ng/mL	0.66 ng/mL	serum	Díaz-Fernández et al. (2019)
DPV	Displacement	ΔPSap4#5	1 pg/mL - 100 ng/mL	0.064 pg/mL	spiked serum	Raouafi et al. (2019)
SWV	Displacement	ΔPSap4#5	10 fg/mL - 100 ng/mL	2.3 fg/mL	spiked serum	Zhao and Ma (2018)

between PSAG-1 reactive PSA and total PSA) it is possible to distinguish between PCa patients from healthy ones or patients with benign prostatic pathology (Díaz-Fernández et al., 2021).

There are other techniques that have been used for direct measurement of PSA using the same redox probe as the impedimetric sensors. For example, a mesoporous silica thin film-coated (MSF) gold electrode was used as a sensing interface. Signal was measured by DPV resulting in high sensitivity, selectivity and a monthly storage stability, maintaining the analytical signal above 97%. In addition, it was validated in human serum as well as in spiked artificial urine samples (Argoubi et al., 2018). A comparison between an immunosensor and an aptasensor was performed in spiked serum samples working with graphene quantum dots-gold nanorods modified screen-printed electrodes and using three electrochemical techniques (CV, EIS and DPV). Both of them show promising results in terms of repeatability and sensitivity. Nevertheless, the aptasensor presents better stability, simplicity and cost effectiveness (Srivastava et al., 2018).

Reagentless direct assays are the ideal formats, and they can be achieved through different approaches. Using aptamers tagged with methylene blue (MB) as electroactive label is the most common one. This approach relies on the conformational change experienced by the aptamer in the presence of the target, which places MB closer or farther from the electrode surface (Fig. 2B). In 2021, three MB-aptamers (all of them based on the ΔPSap4#5 aptamer) with different conformations were compared, including two stem-loop structures and a doble strand structure, using SWV. The aptamer with the greater signal change after PSA incubation was a short single-stranded DNA pseudoknot that forms two stem-loop structures. The remarkable characteristics are that the sensor is effectively regenerated over four uses, it can maintain the 83.2% of the initial signal after 14 days and spiked serum samples have acceptable recoveries (Zhao et al., 2021).

Other redox labels have been used, like hemin (Zhang et al., 2018a) and thionine (Wei et al., 2018), which are incorporated on the nanomaterials that modify the electrode and detected by DPV. The aptasensor based on the use of thionine is constructed on a microfluidic paper device, turning into a low-cost, portable and easy to operate platform, with a good response in human serum samples. The binding of PSA to the aptamer decreases the thionine current. In contrast, in the hemin approach the current increases because the layers on which the aptamer is attached are released from the graphene-palladium nanoparticles support.

Despite direct assays are the most widely used because of their simplicity and ease of implementation in the clinical field, more complex designs have also been proposed. Several sandwich assays were reported using not only aptamers, but also the combination of an antibody as capture receptor and the aptamer as the detection one (Meng et al., 2019). Paying attention to the aptamer-based sensors, the integration of ΔPSap4#5 and PSA-1 aptamers into a sandwich configuration was the first time that PSA was detected focusing on their glycan moiety using aptamers (Fig. 2C). This design is characterized by a LOD of 0.66 ng/mL and a linear range between 0.66 ng/mL and 25 ng/mL in serum samples with a minimal dilution, which encompasses the relevance clinical PSA concentration range. Validation was performed in clinical samples with the standard chemiluminescence ELISA, and the aptasensor provides a good discrimination between PCa and benign prostate hyperplasia (Díaz-Fernández et al., 2019). This indicates that the detection of a still

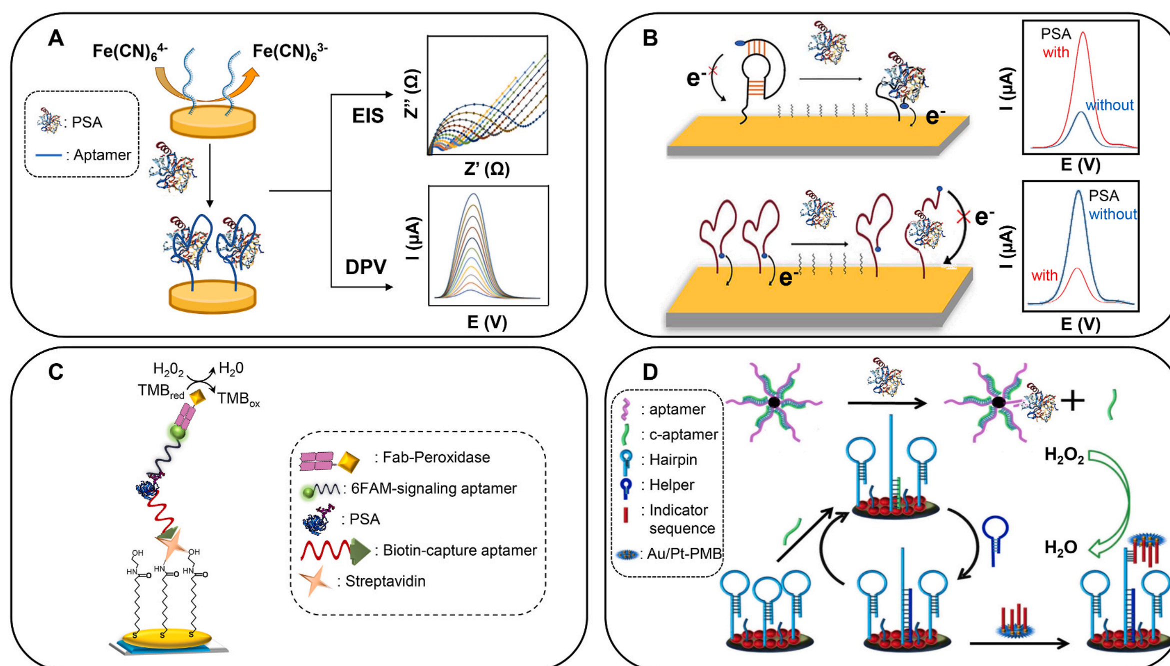

Fig. 2. Electrochemical aptaassays for PSA detection. (A) Label-free direct assay with impedimetric or voltammetric monitoring of the biorecognition event in the presence of ferri/ferrocyanide added to the solution. (B) Reagentless direct assay based on a conformation change undergone by a pseudoknot-like aptamer tagged with an electroactive molecule in the presence of the biomarker. Adapted with permission from (Zhao et al., 2021). (C) Sandwich assay involving a surface-confined capture aptamer against PSA peptide region and a signaling aptamer that binds external sugars in the PSA glycan region. Reprinted with permission from (Díaz-Fernández et al., 2019). (D) PSA-induced catalytic hairpin assembly and Au/Pt-polymethylene blue (PMB) composite attachment. Adapted with permission from (Zhao and Ma, 2018). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

undefined fraction of the glycosylated PSA that responds to PSA-1 aptamer might be of higher clinical validity than the determination of the total PSA.

Another sandwich assay uses a microfluidic chip containing interdigitated electrodes that integrates a dielectrophoretic separation to eliminate the blood cells present in whole blood. In this case apt2 is used as the capture aptamer and ΔPSap4#5 as the detection one. This design allows to determine PSA by DPV directly in whole blood simplifying the operation procedure. Therefore, it is a good candidate for clinical diagnosis as point-of-care testing (POCT) (Wang et al., 2022).

Finally, displacement assays are also possible. An enzyme-free recycling with a target-induce catalytic amplification strategy can improve the detectability. The aptamer is hybridized to a complementary strand (c-aptamer) on magnetic beads. The latter is released when PSA is present and triggers the opening of the hairpin immobilized on the electrode surface. The addition of a helping hairpin displaces the triggering DNA strand initiating a catalytic cycle. Finally, the opened hairpin is detected by an indicator DNA sequence attached to a Pt/Au-polymethylene blue composite (Fig. 2D). In this case, the complexity of the configuration is justified by the exceptional analytical characteristics, in particular, the wide linear range covering seven orders of magnitude and the ultralow LOD of 2.3 fg/mL. In addition, good recoveries (98.8–103.0%) were obtained for spiked serum samples (Zhao and Ma, 2018). Over a functionalized-graphene carbon screen-printed electrodes the ΔPSap4#5 aptamer and a partially complementary sequence were immobilized and MB was intercalated. Once the protein-aptamer reaction occurs, the complementary strand is displaced and the amount of intercalated MB decreases. The current measured by DPV decreases and the peak potential negatively shifts when PSA concentration increases. To achieve the best sensitivity a large aptamer concentration is required (50 μM). The sensitivity is greater and the LOD lower when using the current as the analytical signal. The linear range is suitable to cover clinical needs, selectivity was tested in the presence of major serum proteins and the assay exhibits a high stability and reproducibility. Moreover, spiked serum samples were analyzed, and recoveries ranged from 99 to 104%. The stability of the covalently linked aptamer on graphene is also remarkable. After 10 months of modification the responses to PSA are equivalent to a freshly prepared electrode (Raouafi et al., 2019).

To conclude it is worth noting that despite having described RNA aptamers towards PSA, they are no longer used, and ΔPSap4#5 is by far the most common one. In addition, there is a new group of aptamers that recognize the glycosylated fraction of PSA and has provided promising results in order to overcome the lack of specificity of current PSA assays. Regarding the assay type, a big effort is being made to work with direct biosensors in order to simplify and adapt them to be used in hospital laboratories.

2.3. Aptasensors for circulating tumor cells and extracellular vesicles

Early during the formation of primary tumors, CTCs and EVs are shed into the systemic blood, potentially establishing a pre-metastatic niche and promoting immune evasion. In consequence, they constitute another important source of novel biomarkers for cancer diagnosis, prognosis, and prediction of treatment response.

The detection and molecular characterization of CTCs is a challenging problem, as they occur in the peripheral blood at very low concentrations (an average of between 5 and 50 cancer cells per 7.5 mL of blood in a background of millions of blood cells). Methods currently in use combine isolation/enrichment and detection. Enrichment technologies are based on physical (size, density, electric charges or deformability) or biological (surface protein expression and invasion capacity) differences between blood cells and CTCs (Alix-Panabières and Pantel, 2014). Biological enrichment methods can be easily integrated with biosensor technology. They are mostly based on immunomagnetic separations, using magnetic particles modified with antibodies against different surface proteins, mainly the epithelial cell adhesion molecule (EpCAM), to selectively entrap CTCs. The only method approved by the

US Food and Drug Administration (FDA) to monitor CTCs associated to PCa, CellSearch® (Menarini Silicon Biosystems), is based on this principle.

Alternative receptors for capturing CTCs are aptamers against the same surface proteins used for immunocapturing. Several aptamers, both RNA (Shigdar et al., 2011) and DNA (Alshaer et al., 2017; Song et al., 2013; Zamay et al., 2019), targeting EpCAM have been selected. The first one was an RNA obtained using as target a recombinant form of the C-terminal cytoplasmic domain of EpCAM (Shigdar et al., 2011). One aptamer thus obtained, after optimization by truncation (EpDT3: 5′-GCG ACU GGU UAC CCG GUC G-3′), shows an affinity in the range of 50 nM to different EpCAM-positive cell lines. However, this aptamer is highly prone to endonuclease degradation, and little work exists on their use. Subsequently, the same authors developed another SELEX process versus the recombinant protein using a DNA library (Song et al., 2013). This process led, after optimization, to the aptamer called SYL3C (5′-CAC TAC AGA GGT TGC GTC TGT CCC ACG TTG TCA TGG GGG GTT GGC CTG-3′), capable of recognizing EpCAM-positive cells from a mixture of cells in a culture media, showing an enrichment efficiency for CTCs about 60%. There are various electrochemical aptasensors for the detection of CTCs using the SYL3C aptamer as specific receptor (Hashkavayi et al., 2021; Shen et al., 2019; Zheng et al., 2014). These sensing approaches are based on a classical sandwich design, where either the aptamer (Hashkavayi et al., 2021; Zheng et al., 2014) or an anti-EpCAM antibody (Shen et al., 2019) are immobilized onto a surface for the selective capture of EpCAM-positive cells. A key for maximizing the capture efficiency of these platforms is to optimize the surface concentration of the capture ligand. This is achieved by using as support different nanostructures such as magnetic nanoparticles (Shen et al., 2019) and gold nanoparticles (Zheng et al., 2014) or nanostars (Hashkavayi et al., 2021). The high sensitivity required to detect the extremely low CTC counts in body fluids is achieved through signal amplification: i) using the signaling aptamer conjugated to hybrid nanoparticles with catalytic activity toward the electrochemical reduction of thionine, which is the final readout (Zheng et al., 2014); and ii) exploiting the nucleic acid nature of the signaling ligands, they are elongated by rolling circle amplification, a nucleic acid amplification method that do not require temperature cycling (Hashkavayi et al., 2021; Shen et al., 2019). Both approaches led to a large increase in the final electrochemical signal, rendering such sensors particularly sensitive with LOD in the range 1–5 CTCs in aqueous buffer. Nonetheless, the sensitivity decreases when the sensor is challenged with plasma or whole blood samples spiked with tumor cells (Shen et al., 2019). Though these designs have not yet been used for PCa diagnosis, they are general platforms for EpCAM-positive cells. However, as EpCAM is a cell surface protein overexpressed in different epithelial carcinomas, the use of aptamers targeting a recombinant form of the protein may compromise the selectivity of the sensors. An alternative approach is to obtain aptamers recognizing the native form of the protein on the tumor cells. This was achieved with lung cancer cells, using as target during the SELEX process cells isolated from tumors of patients (Zamay et al., 2019). But, to our knowledge, there are no aptamers evolved against the native form of EpCAM on PCa cells.

Another alternative is to target membrane proteins more specific for PCa cells. This is the case of prostate specific membrane antigen (PSMA), which is overexpressed in prostate tumor cells. There are RNA aptamers that specifically bind to PSMA (Lupold et al., 2002). The truncation of one of these aptamers, through experimentation and logical design, led to a smaller isoform A10 (5′-GGG AGG ACG AUG CGG AUC AGC CAU GUU UAC GUC ACU CCU UGU CAA UCC UCA UCG GC-3′), which contains 2′-fluoropyrimidine (underlined nucleotides) to improve the nuclease stability of the aptamer. An electrochemical impedance sensor for PCa cells was engineered by attaching the biotin-modified A10 aptamer to a gold surface modified with streptavidin (Min et al., 2010). Binding of PSMA(+) PCa cells to the aptamer hinders the electron transfer of the redox probe that must be added to the solution, leading to a readily measurable electrochemical signal. This is a simple approach, distinguishing 10^2 PCa cells in 10^4 normal cells in culture media. However, this platform has not been tested in biological samples, where the high abundance of other cells and proteins could lead to false positive results.

In addition to CTCs, EVs can also be shed from the tumor mass. They are classified according to their size in exosomes, microvesicles, and apoptotic vesicles. Exosomes are nanosized EVs, between 30 and 150 nm in diameter, which are released from cells after the fusion of multivesicular bodies with the plasma membrane. They contain surface proteins similar to cells, and enclose molecules such as proteins, DNA and RNA that are characteristic of the parent cells. Tumor-derived exosomes are actively secreted in larger quantities than CTCs, contributing to tumorigenesis and angiogenic processes. Thus, PCa exosomes in blood or urine have been recognized as promising biomarkers for early PCa diagnosis (Akoto and Sharanjot Saini, 2021).

Commonly, exosomes are firstly isolated and enriched from the biological samples by ultracentrifugation, ultrafiltration, precipitation, or immunoaffinity-based exosome isolation. Afterwards, they are quantified using various techniques; the most popular are nanoparticle tracking analysis (NTA), dynamic light scattering (DLS) or enzyme-linked immunosorbent assays (ELISA) (Chia et al., 2017). These are in general time-consuming and expensive methods, making them difficult to implement in clinical practice. In an attempt to obtain rapid and cost-effective methods for detecting tumor exosomes in body fluids, biosensors are attracting increased attention.

Several issues need to be addressed for designing cancerous exosomes biosensors. First, the exosomal biomarker used as target for biosensor design must be selected. Exosome membranes are composed of a lipid bilayer, which is enriched in cholesterol, phosphatidylserine and ceramide along with tetraspanins, adhesion molecules, proteases, and transmembrane receptors from the precursor cell. Although to detect total exosomes general targets such as tetraspanins (CD9, CD63, CD81, and CD82) or lipids are commonly employed, for the detection of tumor exosomes the use of cancer-associated antigens is preferable. In the particular case of PCa, the following molecules have been identified as specific targets: EpCAM and insulin-like growth factor receptor (IGFR), which are associated with different solid tumors including PCa, and PSMA that is an indicator of PCa (Cheng et al., 2019). Second, a selective ligand for the selected biomarkers is needed. Aptamers are excellent candidates. Finally, to reach the high sensitivity required, a signal amplification strategy is usually employed. To date, the DNA aptamer SZTI01 (5′-GCG TTT TCG CTT TTG CGT TTT GGG TCA TCT GCT TAC GAT AGC AAT GCT-3′), originally selected against a recombinant form of the extracellular domain of PSMA (Boyacioglu et al., 2013), has been used for the design of an electrochemical platform detecting exosomes derived from LNCaP cells, a cell line derived from a metastatic lymph node lesion of human prostate cancer (Dong et al., 2018). It is based on a magneto-electrochemical assay, where in a first step the exosomes are magnetically captured by the aptamer immobilized onto magnetic microparticles. The aptamer is initially hybridized with three different complementary DNA oligonucleotides, and the exosome binding induces their release. Thus, exosome quantification is converted into oligonucleotide detection (Fig. 3). This is done by a hybridization assay on gold electrodes, combined with exonuclease III-based target recycling. The final electrochemical response is obtained using $Ru(NH_3)_6^{3+}$ as electroactive probe, taking advantage of its electrostatic interaction with the surface DNA. The platform leads to a decreasing signal for increasing amounts of exosomes in culture media, with a LOD of 70 particles/μL. Translating this system into routine clinical tests, however, is still limited by i) the slow response, the assay takes more than 5 h to deliver the final readout, ii) exosome isolation and detection are not integrated, and iii) its analytical performance has only been demonstrated in fetal bovine serum spiked with exosomes isolated from a culture medium.

A nice example of the power of the negative selection step in SELEX

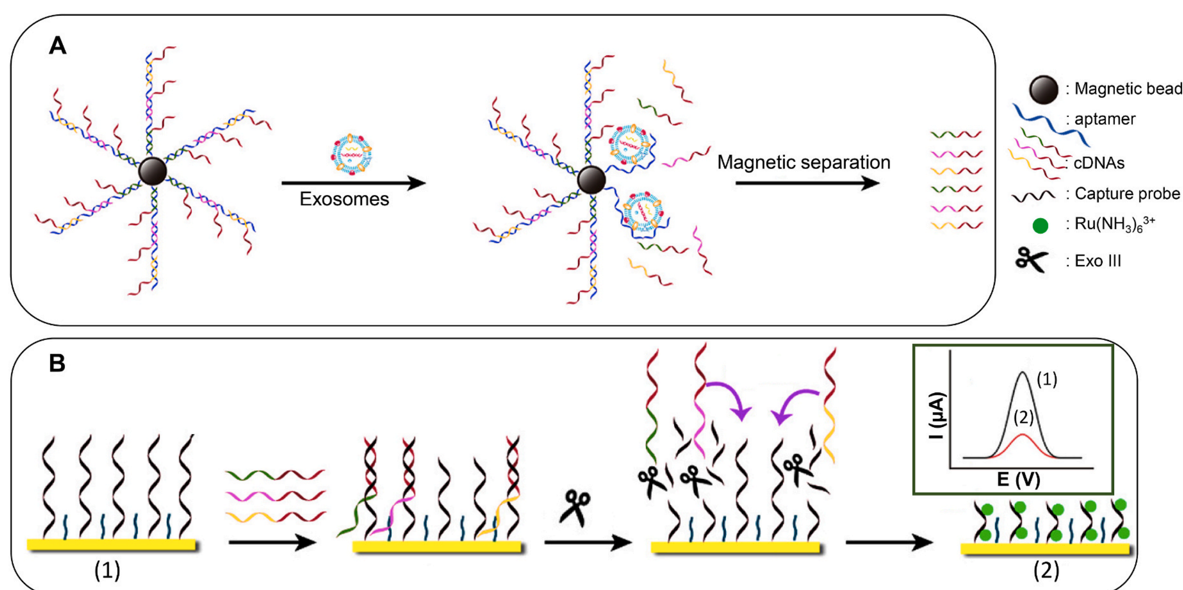

Fig. 3. Electrochemical aptaassay for exosomes detection. (A) Exosomes are captured by aptamers anchored onto magnetic beads with concomitant release of DNA probes complementary to the bioreceptors (c-DNA). (B) Detection of c-DNA by hybridization onto the transducer surface, Exo III cyclic digestion and final measurement of $Ru(NH_3)_6^{3+}$ electrostatically immobilized. Adapted with permission from (Dong et al., 2018).

to obtain aptamers with the desired selectivity for a certain condition is the selection of aptamers that discriminates exosomes secreted by different PCa cell lines. The target that allowed the capture of VCaP but not LNCaP cells was identified as YBX1 by MS and additional ELONA assays. The best aptamer called sequence 7 (5′- TAC TTA ATT GGG GGG GGG GAT TCT GTT TTG TCT CT-3′) expands the very limited number of aptamers available for the isolation of a specific type of exosomes (Hornung et al., 2020).

2.4. Aptasensors for other emerging PCa biomarkers

The active search for replacing PSA as PCa biomarker has found several candidates though they still remain as candidates and are far from being implemented in the clinics. As expected, aptamers for these biomarkers that include proteins, amino acids or even RNA transcripts have been selected. In this section we discuss those approaches whose aptamer is traceable to the original SELEX.

In 2009, sarcosine, an amino acid analogue, was proposed as a biomarker for both early PCa detection and prediction of aggressiveness. Its level was found increased in urine (sediment or supernatant) in biopsy positive males and its predictive value overpassed the one of PSA when restricted to the PSA grey zone. Besides, its addition to benign prostate epithelial cells transformed their phenotype into an invasive one (Sreekumar et al., 2009). Since then, its role as a PCa biomarker has been debatable and several works advice against its usefulness both in urine (Gkotsos et al., 2017; Jentzmik et al., 2010; Struys et al., 2010) and serum samples (Ankerst et al., 2015).

Nonetheless, there are two aptamers derived against sarcosine using two opposite strategies. In the first one the oligonucleotide library is immobilized through hybridization to agarose beads and the analyte in solution displaces the sequences with high affinity from the duplex (Luo et al., 2018). The second one is performed in solution and the separation is achieved thanks to the preferential binding of free ssDNA to graphene oxide (GO) (Özyurt et al., 2019). The output of the GO-SELEX were two sequences, 9S (5′- TAG GGA AGA GAA GGA CAT ATG ATG TGC CGC GCT TCC CTT GCC GCT CAA AAC AGA CCA CCC ACT TTG ACT AGT ACA TGA CCA CTT GA-3’) and 13S (5′- TAG GGA AGA GAA GGA CAT ATG ATG TGT TGT TCA GCC GCT ACT ACT TCC CTT CCA GTT TAA CGT TTG ACT AGT ACA TGA CCA CTT GA-3′), with impressively low dissociation constants 0.33 and 5.6 nM for a very small molecule. In contrast, sar11 (5′- CGG GAC GAC CAC GCA AAT ACG AAT AGT GTG AAC GCG GGA GTC CCG-3′) presents a K_d of 135 nM. This difference might be explained by the use of counter steps in the GO-SELEX. All constants were estimated by fluorescence using different formats. Only the sar11 was used with analytical purposes in a displacement self-indicating assay. Interestingly the aptamer is coupled to a graphene mimicking nanomaterial, MoS_2, through a poly-C tail and used in a dual sensor for the simultaneous detection of PSA. The aptamer is partially hybridized to a DNA strand covalently linked to silica nanoparticles bearing a large number of ferrocene molecules (for sarcosine) or MB (for PSA). The extremely low sensitivity for such a small molecule (in the fg/mL range) is attributed to this amplification. For PSA, it was estimated in a 1000-fold improvement in comparison with a similar assay using the redox-tagged complementary strand alone without nanoparticles. (Yan et al., 2022).

Alpha-methylacyl-CoA racemase (AMACR or P504S) is a mitochondrial enzyme that can be found in body fluids. Its expression in tissues is increased in PCa and other tumors. A meta-analysis correlates its level with high PCa risk (Jiang et al., 2013). Because of the high cost of the his-tag recombinant AMACR, a single-bead SELEX was performed that required just 45 ng in 5 rounds of selection. The wining sequence AMC51 (5′-CCC TAC GGC GCT AAC CCA TGC TAC GAA TTC GTT GTT AAA CAA TAG GCC ACC GTG CTA CAA-3′) was used in a direct voltametric assay. The sensing phase containing polypyrrol and PEG was designed to avoid electrode fouling. The unusual polyhistidine tagged aptamer AMC51 was anchored through a Cu^{2+} chelate complex. The recognition of AMACR was monitored by the decrease in the SWV current of the Cu^{2+}/Cu^{+} redox process. Under optimized conditions 5 fM of AMACR was reliably detected in diluted spiked human plasma samples (Jolly et al., 2016a). A his-tagged recombinant protein that can also form a chelate with the sensing phase was used, which cannot allow the verification of the aptamer ability to recognize the natural AMACR present in blood.

Engrailed 2 (EN2) protein might be an ideal urinary biomarker of PCa because non-cancerous prostatic cells (normal or hypertrophic) do not secrete it. High levels of EN2 have been found in urine of PCa patients and exogenous addition to prostate cell lines stimulates different phenotypes related with tumorigenic capacity (Gómez-Gómez et al., 2019). However, its increase is not exclusive of PCa. A meta-analysis confirms a high specificity but a low sensitivity of EN2 as a biomarker

in urine (Da Rosa et al., 2017). The DNA sequence used for its determination has not been obtained through SELEX but rationally engineered. The designed sequence is a hairpin that contains the specific binding site TAATTA that EN2 binds to regulate transcription. Using this DNA sequence, (5′-CGT GTA ATT ACC TCC AGA AGG AGA GGT AAT TAC ACG-3', the underlined nucleotides are the EN2 binding site) EN2 was determined in artificial urine by a direct impedimetric assay achieving an extremely low limit of detection of about 6 fM (Lee et al., 2015). Another work employs cyclic voltammetry and ferricyanide as redox probe. As expected, the detectability is much poorer (nM) and it was not tested in body fluids (Settu et al., 2017).

It is valuable to discuss the case of an aptamer selected toward a lncRNA target, the PCA3, which exhibits a very strong secondary structure arising from its long size (3923 nt). The actual target is a small region of 227 nt that was directly converted into DNA because of the RNA instability to perform the SELEX. Authors recognize that the folding of RNA might not be equal to DNA folding so the target might have a different conformation. Nonetheless, they claim the specific binding of the aptamer CG3 (5′-AGU UUU UGC GUG UGC CCU UUU UGU CCC C-3′) to the folded RNA and not to denatured RNA or DNA (Marangoni et al., 2015). However, it is unclear how denatured RNA was tested because at high temperatures binding is not expected. Closer inspection of the aptamer CG3 clearly shows its complementarity with the target, which argues against structural recognition. The poor analytical performance

Table 2
Characteristics of the reported genosensors for the detection of miRNA associated to PCa.

Analyte	Sensing platform	Assay Format	Transduction	Amplification	Dynamic range & LOD	Sample	Ref.
miR-21	GC/H_2N-naphthalene-SO_2/CP	Direct (Signal-off)	Coulometry with $[Fe(CN)_6]^{3-/4-}$	_ _	10 fM-10 nM 20 fM	Spiked human urine	Smith et al. (2017)
miR-429	SOI/NW/CP	Direct (Signal-off)	Conductance	_ _	0.33 fM-0.33pM 0.33 fM	RNA from plasma (2 PCa patients + 1 volunteer)	Sabahi et al. (2020)
miR-410	PGE/PNT-AuNP/CP	Direct (Signal-on)	EIS with $[Fe(CN)_6]^{3-/4-}$	_ _	10 fM-300 pM 3.90 fM	Spiked 1:1 diluted serum (Recovery 98–109%)	Yaman et al. (2020)
miR-21 miR-1246 Let7b	ITO/SnO_2/rGON//PANHS/PNA	Direct	FET (ΔVoltage)	_ _	10 fM-10 nM 10 fM	Urine of PCa (6) and healthy individuals (4)	Kim et al. (2021)
miR-21	FTO/SWCNT/denAu//PNA	Direct (Signal-on)	DPV of Cd^{2+} electrostatic and covalently bound to the target	_ _	0.01fM-1μM 0.01 fM	Spiked diluted serum (Recovery 97–107%)	Ivanov et al. (2019)
miR-375	AuE/mSAM (HS-CP + mercapto-succinic acid)	Direct (Signal-off)	SWV of $[Fe(CN)_6]^{3-/4-}$	_ _	10 aM-1 nM 11.7 aM (buffer) 12.4 aM (serum)	Spiked 100-fold diluted serum	Jeong et al. (2019)
miR-145 (suppressor)	AuE/PNA + MCH	Direct	(a) EIS non Faradaic (b) SWV of Fc	AuNPs modified with PEI	1 fM-100 nM 0.37 fM	Spiked buffer	Jolly et al. (2016b)
miR-21	Biotin-HpCP-AuNP	Direct (Signal-on)	SSWV of Au^{3+} from oxidation of AuNPs in acid	Silver deposition onto AuNP	10 fM-10 nM 4 fM	Spiked serum	Fredj et al. (2017)
miR-155	MP/CP	Sandwich	DPV of 1-naphthol enzymatically generated	Alkaline phosphatase	81 pM-1.2 nM 29 pM	Spiked serum	Mohammadi and Amine (2018)
miR-141 miR-21 let-7a	Homogeneous hybridization & anchor onto AuE-FNAs	One-step sandwich in solution	Amperometry (TMB + H_2O_2)	Peroxidase	1 aM-10 nM 1aM	Total RNA from human PCa cells & normal prostatic stromal myofibroblast cell	Wen et al. (2020)
miR-141	MP/ternary DNA hybrid	Recognition & amplification onto magnetic particles	Photoelectrochemistry (AuNP-based quenching of Pdots/ITO)	Target recycling (Entropy-driven toehold-mediated DNA strand displacement)	1 fM-10 pM 0.5 fM	Spiked blood with RNAse inhibitor (Recovery: 96–108%)	Zhang et al. (2018b)
miR-141	Functional Hp DNA in solution	Direct	SWV of MB free or complexed with G-triplex	λ-exonuclease-assisted target recycling	100 fM –1 nM 16 fM	Spiked 100-fold diluted serum (recovery 92–93%)	Meng et al. (2021)
miR-182-5p	GC/NanoAu/PAMAM-CNTs-Pt/CB-8-MV^{2+}	Host-guest recognition of Trp-DNA surrogate	SWV of $[Ru(NH_3)_6]^{3+}$	Target recycling and SDR followed by heterogeneous HCR	1 fM-500 pM 0.5 fM	Spiked in buffer	Chang et al. (2017)
miR-200c	Homogeneous recognition with two probes partially complementary	Recognition & amplification in solution	SWV of MB	Cascade displacement amplification + heterogeneous SDR	10 aM-10 pM 3.3 aM	Spiked 10-fold diluted serum	Jiang et al. (2021b)

CP: capture probe, *HpCP*: hairpin capture probe, *PNA*: peptide nucleic acid, *FNAs*: framework nucleic acids (tetrahedrons), *AuNP*: gold nanoparticles, *denAu*: dendritic gold nanostructures, *FTO*: fluorine-doped tin oxide, *GC*: glassy carbon, *GO*: graphene oxide, *ITO*: indium tin oxide, *MP*: magnetic particles, *PGE*: pencil graphite electrode, *rGON*: reduced graphene oxide nanosheet, *SOI*: silicon-on-insulator, *MCH*: 6-mercapto-1-hexanol, *mSAM*: mixed self-assembled monolayer, *NW*: nanowire, *PANHS*: pyrene butiric acid N-hydroxysuccinimide ester, *PEI*: poly(ethylenimine), *PNT*: peptide nanotubes, *SWCNTs*: single-walled carbon nanotubes, *Trp*: tryptophan, *SDR*: strand displacement reaction, *HCR*: hybridization chain reaction, *Fc*: ferrocene, *MB*: methylene blue, *MV^{2+}*: methyl viologen, *TMB*: 3,3′,5,5′-tetramethylbenzidine, *DPV*: differential pulse voltammetry, *FET*: Field effect transistor, *SWV*: square wave voltammetry, *SSWV*: stripping square wave voltammetry.

in two direct electrochemical approaches (Nabok et al., 2021; Takita and Nabok, 2021) shed doubts on the suitability of this RNA aptamer.

3. Genosensors for circulating nucleic acids

3.1. miRNA

MicroRNAs or miRNAs are small noncoding RNA molecules with important roles in human gene expression control, particularly, in those genes involved in the onset and development of cancer. Altered expression levels (upregulation or downregulation) of some miRNAs have been associated with different types of cancer including prostate cancer, and numerous evidences point to their significance as diagnostic prostate tumor biomarkers (Andl et al., 2020). Despite their promising role in the early detection of PCa, their profiling and quantification in human body fluids such as serum, plasma or urine present important challenges derived from their low abundance, short length and highly homologous sequences. MiRNAs and other circulating RNAs are conventionally analyzed by northern blot, reverse transcription quantitative polymerase chain reaction (RT-qPCR), hybridization-based microarrays, and RNA sequencing (Ouyang et al., 2019). However, these methods are restricted to central labs with advanced equipment and experienced personnel, and they are therefore not practical for screening studies. The intrinsic properties of electrochemical biosensing platforms turn them into appealing alternatives as summarized in Table 2 and discussed below.

3.1.1. Label-free (& amplification-free) approaches

The simplest approaches for electrochemical biosensing of microRNAs are those that exploit the changes in the electrical properties of the electrode-solution interface derived from the hybridization event between the target microRNA and its complementary DNA sequence, frequently referred to as capture probe (CP).

Thus, for example, the formation of DNA/miRNA hybrids onto glassy carbon electrodes functionalized with amine-tagged DNA capture probes via sulfamide coupling reaction was revealed by coulometry in the presence of $[Fe(CN)_6]^{3-/4-}$. As a result, miR-21 was detected down to 10 fM without the need for labels. Moreover, this genosensor was challenged with fortified urine specimens after a treatment with proteinase K and spin-filtration to remove proteins, thereby preventing electrode fouling. The results were in good agreement with those obtained with the standard method RT-qPCR (Smith et al., 2017). Similarly, in the category of label-free electrochemical detection methods, nanowire (NW) biosensors based on silicon-on-insulator (SOI) structures, with n-type conductance allow highly sensitive real-time monitoring of DNA-based analogues of miRNAs at subfemtomolar level (about 10^5 molecules in 450 μL). Likewise, discrimination between plasma samples from PCa patients and healthy individuals was attained after miRNA isolation with a commercial kit (Ivanov et al., 2019).

The impedance changes after specific capture of the target miRNA onto the electrode surface via hybridization have been also evaluated. With the aim of improving the electrochemical detection, either by increasing the surface area and in turn the number of immobilized bioreceptors, or by enhancing electrical properties, nanostructured materials have been widely used in impedimetric genosensing of miRNAs. In this regard, peptide nanotubes (PNTs) arising from a self-assembly process of diphenylalanine peptide have been combined with gold nanoparticles and then immobilized onto pencil graphite electrodes for the attachment of HS-DNA CP (Yaman et al., 2020). The resulting nanoarchitectures were applied for impedimetric analysis of miR-410 in spiked diluted serum with recoveries higher than 90% for femtomolar concentrations.

Special mention deserves the analytical device developed by Lee and collaborators (Kim et al., 2021). It is a field-effect transistor (FET) biosensor that combines disposable chips modified with reduced graphene oxide nanosheet (rGON) along with peptide nucleic acids (PNAs) for entrapping miRNAs upregulated in urine of PCa patients. The synthetic, uncharged DNA analogues, i.e. the PNAs, exhibit better stability and selectivity of hybridization than their biological counterparts. Furthermore, enhanced surface area and conductivity are provided by rGON leading to larger voltage variations. This way, simultaneous detection of miR-21, let7b, and miR-1246 in untreated urine was carried out in just 20 min and without signal amplification. The results recorded for prostate cancer and noncancer urine were supported by pathological diagnosis, even if PSA levels matched the grey zone.

Since microRNAs are polyanions, their electrostatic interaction with a positively charged redox probe can be employed for electrochemical sensing. In order to maximize the signal variation in the presence of miR-21, a complementary PNA probe was immobilized onto the surface of fluorine-doped tin oxide electrodes previously functionalized with single-walled carbon nanotubes (SWCNTs) and dendritic gold nanostructures (den-Au), thus attaining larger effective area and conductivity. The generation of the PNA-miRNA duplex onto the transducer promotes the binding of Cd(II) to the phosphate backbone of miR-21, which is subsequently determined by differential pulse voltammetry (DPV). This assay led to the lowest detectability reported with a label-free electrochemical genosensing platform for miRNA analysis (LOD = 10 aM), and it was tested in spiked human serum, finding high recovery percentages (Sabahi et al., 2020).

Curiously, a very close detection limit for miR-375 was achieved in spiked 100-fold diluted serum (12.4 aM) with an approach apparently simpler (Jeong et al., 2019). It is relied on a mixed self-assembled monolayer consisting of a HS-DNA capture probe and mercaptosuccinic acid as blocking agent, built onto screen-printed gold electrodes. Quantification of miR-375 is then performed by square wave voltammetry (SWV) with $[Fe(CN)_6]^{3-/4-}$ in solution (signal-off assay). Such an ultralow detectability (close to 10^{-19} M without dilutions) cannot be attributed either to the use of nanomaterials or to synthetic DNA analogues as capture probes.

3.1.2. Signal amplification approaches

As mentioned above, the low abundance of miRNAs in human body fluids makes the analysis of these potential PCa biomarkers challenging, especially for those serving as tumor suppressors (underexpressed in cancer). To circumvent this drawback, different approaches involving one or several amplification steps have been proposed.

For instance, a simple amplification strategy for impedimetric detection of miR-145, which is downregulated in prostate cancer, has been developed by using gold nanoparticles (AuNPs) modified with poly (ethylenimine). These positively charged labels bind to the PNA/miR-145 duplexes formed onto the transducer surface upon the molecular recognition event, giving rise to a change in the capacitance monitored by EIS in non-faradaic mode. As a double check to exclude false positives, complementary SWV measurements of thiolated ferrocene chemisorbed onto the electrostatically bound AuNPs were performed as well (Jolly et al., 2016b).

A 25-fold increase in detectability (from 0.1 pM to 4 fM) was accomplished by implementing metallic silver deposition onto AuNPs (Fig. 4A). The recognition element is a hairpin capture probe (HpCP) modified at its 5′ and 3’ ends with biotin and AuNP, respectively. In the presence of the target, miR-21, the hairpin opens, making accessible the biotin for binding onto neutravidin-coated glassy carbon electrodes. The AuNPs are detected by stripping square wave voltammetry, resulting in a LOD of 0.1 pM. Further amplification is achieved by silver deposition and subsequent detection by stripping voltammetry. The method feasibility was tested in spiked human serum, finding an average yield of 93% (Fredj et al., 2017).

The use of redox enzymes for electrochemical signal amplification has been also described in the context of miRNA determination. Alkaline phosphatase (AP) was employed to label the DNA/miRNA duplex resulting from a sandwich-type genoassay developed onto magnetic microparticles by means of the widely used biotin-streptavidin affinity

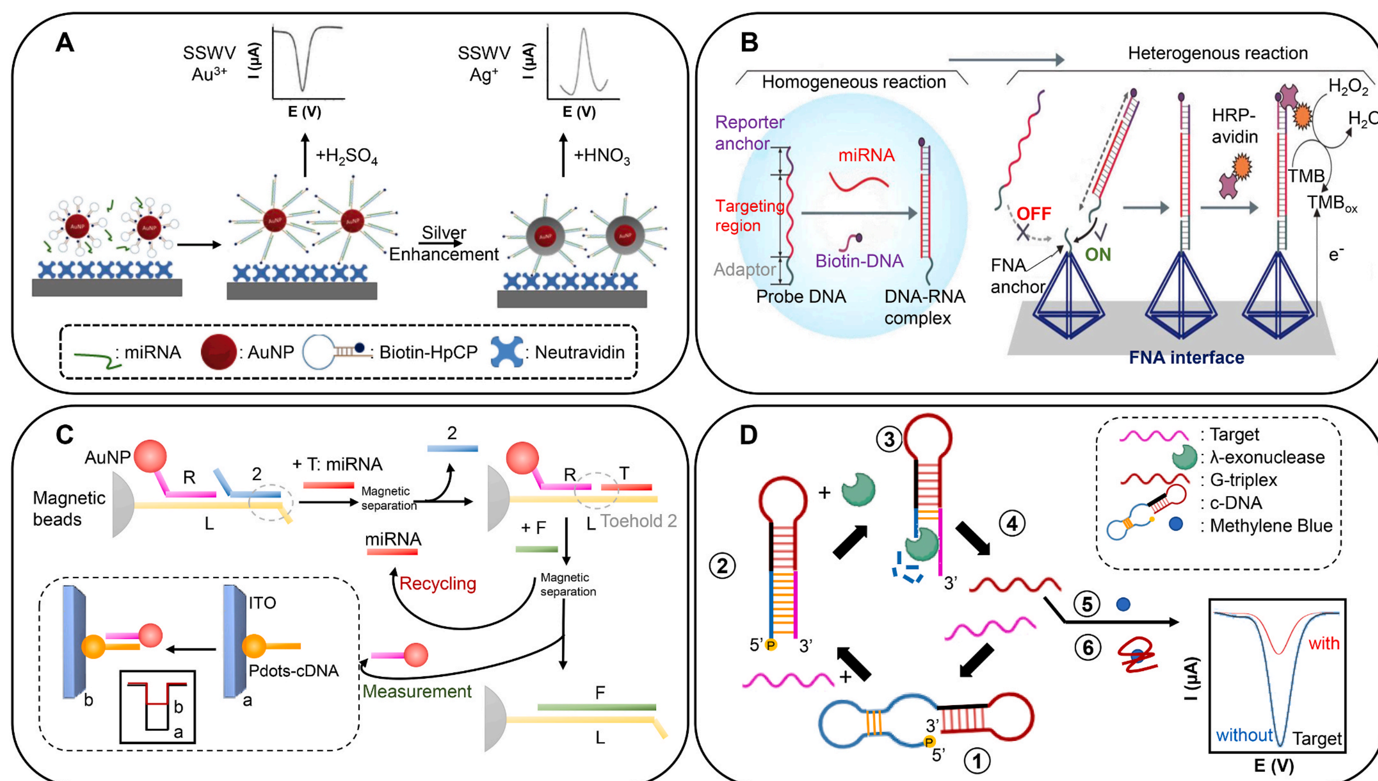

Fig. 4. Signal amplification strategies for electrochemical genosensing of PCa-associated miRNAs. (A) Silver deposition onto gold nanoparticles linked to specific duplexes, followed by stripping square wave voltammetry (SSWV). Adapted with permission from (Fredj et al., 2017) (B) Enzyme-based signal amplification to reveal the generation of ternary duplexes in solution and the anchoring onto the transducer surface functionalized with framework nucleic acids (FNAs). Reprinted with permission from (Wen et al., 2020). (C) Target recycling entropically driven onto magnetic particles and release of AuNP reporter strands for photocurrent quenching. Adapted with permission from (Zhang et al., 2018a). (D) Digestion of DNA/miRNA hybrids with λ-exonuclease induces target recycling and displacement of G-triplex that binds the electroactive molecule methylene blue. Reprinted from (Meng et al., 2021) under a Creative Common Attribution Licence. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

reaction. Then, the AP activity specifically bound to the magnetic scaffold was quantified by differential pulse voltammetry (DPV) of 1-naphthol enzymatically generated. This method was applied to detect miR-155 in human serum at picomolar concentration (Mohammadi and Amine, 2018). A boost in sensitivity could be envisaged by substituting the streptavidin-AP conjugate with unfavorable 2:1 stoichiometry by a monovalent conjugate enzyme based on antibody Fab fragments (e.g. antifluorescein Fab-AP, 1:1) to guarantee one AP molecule per hybridization event (González-Fernández et al., 2013).

As a consequence of its large turnover number, the enzyme peroxidase (POD) is frequently used as the label in sandwich format genoassays. Particularly, a two-phase biosensing strategy involving POD was developed for simultaneous determination of several miRNAs. First, a DNA probe complementary to the target and flanked by two regions, one for reporting and the other one for anchoring, is hybridized in solution together with a biotinylated reporter strand and the target miRNA. The resulting biotinylated ternary duplex is then attached to the transducer surface functionalized with DNA tetrahedral nanostructures by hybridization with the anchor sequence of the probe (Fig. 4B). Next, the avidin-POD conjugate is incorporated and, after enzyme reaction in the presence of the substrates H_2O_2 and 3,3′,5,5′-tetramethylbenzidine, the electrocatalytic reduction current is measured by amperometry (Wen et al., 2020). This approach presents a wide dynamic response range and very low detectability (response from 1 aM to 10 nM, and LOD = 1 aM, respectively). It has been successfully implemented in 16 × sensor chips, thus allowing the detection of three PCa-related miRNAs: miR-141, miR-21, and let-7a. Moreover, its capability to detect the miRNAs within the total RNA extracted from PCa cell lines has been demonstrated. Although more demanding in terms of detectability, its extension to the analysis of circulating miRNAs in human body fluids would be of great interest.

Amplification of the electrochemical signal recorded with miRNA sensing platforms can be also accomplished by target recycling implementation. In this strategy each target miRNA molecule participates in multiple biorecognition events, thus providing a boost in sensitivity. The release of the target molecule from the initially formed duplex to participate in further hybridization events can be thermodynamically driven or assisted by nucleic acid modifying enzymes.

Entropy-driven target recycling is framed within the first group. In general, a fuel strand (F) reacts with a three-stranded complex, displacing the target and reporter strands (T and R) from the linker strand (L) (Fig. 4C). Since the number of base pairs in reactants and products does not change (enthalpy variation is negligible), the reaction is driven by the increment in entropy (Zhang et al., 2007). This strategy has been developed onto magnetic particles for the analysis of miR-141 in spiked undiluted blood samples, thus minimizing the negative effects of this complex matrix. Moreover, the reporter strand (R) was labeled with AuNPs, which acts as a quencher of a photoactive material (photoactive polymer dots or Pdots) covering the surface of an ITO electrode (Fig. 4C). As a result, a concentration-dependent photocurrent decrease was recorded from 1 fM to 10 pM (Zhang et al., 2018b).

However, most of the assays based on target recycling include nucleic acid modifying enzymes that work in solution at mild conditions. Some of them recognize a specific sequence in the nucleic acid substrate, while others enable more general designs. In this vein, a microRNA biosensing assay where target recycling is assisted by λ-exonuclease has been recently reported (Meng et al., 2021). This nucleic acid modifying enzyme catalyzes the removal of nucleotides from the 5′-phosphorylated

end of double-stranded nucleic acids irrespective of the sequence. The presence of the target miRNA triggers the opening of a hairpin biorecognition probe harboring a G-triplex sequence and the complementary fragment to the miRNA. The resulting double-stranded structure is digested by λ-exonuclease with the concomitant release of the intact target, for a new catalytic cycle, and the G-triplex that binds the MB redox probe in solution. As a consequence, the diffusion current of MB decreases and is used as the final readout (Fig. 4D). The method exhibits a dynamic response range from 100 fM to 1 nM. Despite these features, detection of miRNA in 100-fold diluted serum was tested at the upper level (10 pM).

More tricky designs have been reported as well. Thus, the combination of a target recycling process involving a DNA polymerase, with a strand displacement reaction involving a nicking endonuclease, produces a large amount of tryptophan (trp)-modified single-stranded DNA fragments. These surrogates are specifically trapped by a cucurbit[8]uril methyl viologen complex (CB-8-MV^{2+}) incorporated onto the surface of a glassy carbon electrode modified with a conducting nanocomposite coating. Finally, heterogeneous hybridization chain reaction (HCR) is carried out for electrostatic immobilization of $[Ru(NH_3)_6]^{3+}$, which is quantified by SWV (Chang et al., 2017). Such an intricate method allows the detection of miR-182-5p at concentration levels between 1 fM and 500 pM. Regeneration of the modified electrode was successfully achieved by electrochemical reduction of methyl viologen (MV^{2+}), thus destructing the ternary complex CB-8- MV^{2+}-Trp. In the context of PCa screening, reuse of the sensing platform would not be an essential attribute; although, given the complexity of the proposed test, it is undoubtedly positive.

Likewise, the determination of miR-200c has been carried out by ligation-dependent cascade strand displacement amplification (SDA) with target recycling assisted by a DNA polymerase. The DNA fragment generated in solution triggers an on-surface SDA. As a result, numerous DNA probes functionalized with methylene blue are immobilized by hybridization onto a mixed self-assembled monolayer built onto gold surfaces. The values obtained in 10-fold diluted serum specimens fortified with miR-200c within the interval of 100 aM to 10 pM were in good agreement with those measured in phosphate buffer (Jiang et al., 2021b). Regeneration of the modified gold surface for additional tests was reported by heating at 94 °C for 10 min in a buffer solution containing tris(2-carboxyethyl)phosphine or TCEP, without affecting the Au–S bond.

3.2. lncRNA

The fraction of the human noncoding transcriptome greater than 200 nucleotides in length, the so-called long noncoding RNAs or lncRNAs, has also become a source of potential biomarkers for prostate cancer. Thus, special attention has been paid to prostate cancer antigen 3 or PCA3, whose overexpression allows prostate cancer to be reliably discriminated from benign (non-cancerous) conditions of the prostate gland (Sartori and Chan, 2014).

The US FDA approved in 2012 a urinary test for PCA3 quantitation that combines isothermal transcription mediated amplification (TMA) with chemiluminescence. This way, a PCA3 score resulting from normalization of PCA3 levels with respect to those of PSA mRNA was defined (Groskopf et al., 2006). This assay has not, however, been widely implemented in clinical labs, mainly due to its complexity and cost.

Alternatively, several electrochemical biosensing platforms have been proposed for quantification of this transcript. Among them, Oliveira Jr. and collaborators developed two impedimetric direct hybridization-based assays (Rodrigues et al., 2021; Soares et al., 2019). They took advantage of the layer-by-layer technology to deposit a nanostructured film onto the electrode surface for subsequent attachment of a PCA3 complementary DNA probe. The combination of chitosan and carbon nanotubes onto gold interdigitated electrodes led to a limit of detection of 128 pM by using a 21-mer synthetic DNA target (Soares et al., 2019), while a slight improvement in detectability (83 pM) was accomplished incorporating gold nanoparticles and chondroitin sulfate onto carbon surfaces (Rodrigues et al., 2021). These platforms were capable of differentiating PCA3-positive from PCA3-negative cell lines.

Although the previous electrochemical approaches just tackle PCA3 determination, with the aim of excluding non-cancer related variations, levels of PCA3 should be normalized to an internal control of stable expression, which demands the development of multianalyte tests. In this context, a dual sandwich hybridization-based chronoamperometric genosensor has been developed for quantification of PCA3, with PSA mRNA serving as endogenous control. This genoassay exploits the large size of PCA3 to incorporate multiple redox enzymes per binding event, making use of several hybridization signaling probes. As a result, both transcripts, PCA3 lncRNA and PSA mRNA, were simultaneously detected at low-picomolar levels. Likewise, capture of the analytes onto magnetic particles functionalized with specific DNA baits, followed by thermal elution and preconcentration for subsequent analysis with the dual genosensor enabled relative PCA3 quantification in the human prostate cancer cell line LNCaP as well as in urine from patients with positive prostate biopsy (Sánchez-Salcedo et al., 2021). Furthermore, to favor its translation to the clinical practice, authors combined the FDA-approved personal glucose meter acting as the electrochemical transducer with magnetic particles as a solid support for the sandwich genoassay. The conversion of PCA3 into glucose concentration was conducted by using alkaline phosphatase, which catalyzes the hydrolysis of D-glucose-1-phosphate into D-glucose (Abardía-Serrano et al., 2020).

Isothermal nucleic acid amplification techniques were also coupled to electrochemical detection for evaluation of PCA3 expression. In this regard, amplicons generated by loop mediated amplification (LAMP) containing digoxigenin-uracyl were entrapped onto magnetic beads by hybridization with surface-bound capture probes, and subsequently labeled with antidigoxigenin-peroxidase. The modified beads were next magnetically captured onto disposable carbon electrodes for measuring the immobilized enzymatic activity in the presence of H_2O_2 and hydroquinone. This methodology allowed the quantification of PCA3 with respect to PSA mRNA both in PCa cell lines and in urine samples from PCa patients and healthy people. Likewise, a tentative PCA3/PSA ratio cut-off was proposed for PCa detection (Moranova et al., 2022).

Faster nucleic acid amplification at lower temperature and involving less primers can be carried out by recombinase polymerase amplification (RPA). This convenient target amplification strategy was conducted onto gold electrodes modified with a nanocomposite of ZnSe nanosheets and gold nanoparticles, where the thiolated forward primer is chemisorbed. The use of a biotinylated reverse primer allowed the incorporation of the streptavidin-alkaline phosphatase conjugate that catalyzes the hydrolysis of *p*-aminophenyl phosphate into *p*-aminophenol. This phenol derivative along with methylene blue act as electron acceptors promoting photoelectrochemical detection without external voltage (self-powered photoelectrochemistry). As a result, attomolar concentrations of PCA3 and KK2 can be detected, even in fortified 10-fold diluted serum, although no attempts to relative PCA3 quantification were described (Hun and Meng, 2020).

3.3. ctDNA

The enzyme-catalyzed addition of a methyl group to the 5′-carbon of cytosine nucleobase at DNA regions involved in regulatory roles, the so-called DNA methylation, is one of the most studied epigenetic modifications involved in cancer development, also in PCa. It is usually detected by methylation-specific PCR (MSP). In this method, methylated and unmethylated cytosines are differentiated after treatment with sodium bisulfite which leads to deamination of unmethylated cytosines into uracils, while methylated counterparts remain unaltered. Then, specific amplification of the modified DNA is achieved by using

appropriate primer sets. However, quantitation of cell-free DNA methylation present in human biofluids at very low concentration levels usually requires two consecutive amplifications (nested-MSP).

For the sake of simplicity, Fan and collaborators developed an electrochemical platform based on DNA nanotetraedrons to capture labeled MSP-resulting amplicons and their subsequent determination by chronoamperometry. The sensing phase architecture provides more controlled orientation and spacing, and in turn enhanced sensitivity. This approach was applied to the analysis of promoter hypermethylation of two prostate cancer-related genes, *GSTP1* and *TNFRSF10D*, in serum samples from PCa and BPH patients as well as healthy individuals (60 samples in total). The combination of both biomarkers showed good agreement between methylation levels and disease, with better discrimination between PCa and benign prostate hyperplasia (BPH) than the serum PSA test (Chen et al., 2020).

In an attempt to circumvent the nucleic acid amplification step, a preconcentration process based on ion concentration polarization (ICP) has been integrated along with a differential pulse voltammetric genosensor into a microfluidic chip (Hong et al., 2018). The ability to detect a random methylated DNA fragment in buffer solution at the femtomolar level was reported as a result of combining a 100-fold preconcentration factor, arising from the nanoelectrokinetic technology, and a 20-fold increase in genosensor's detectability ascribed to the electrode nanostructuring. A proof-of-concept test was then carried out with methylated genes *GSTP1* and *EFEMP1* spiked in urine samples. Despite the conductivity of undiluted urine negatively impacts the ICP phenomenon, both targets were detected down to low picomolar concentrations in just 2 h.

For cancers other than prostate, hybridization-based approaches involving DNA probes complementary to the methylated target and antibodies that specifically recognize methylated cytosine have been also developed. They have demonstrated good performance when faced with genomic DNA (gDNA) extracted from tumoral cell lines or tissues, particularly when incorporating multienzymatic reagents (Povedano et al., 2020). It would be interesting to explore their capability to detect circulating methylated DNA in human body fluids for early (prostate) cancer diagnosis.

Although not a biosensor, it is also worth highlighting a general cancer detection strategy based on aberrant DNA methylation recently published (Sina et al., 2018). It exploits the differential adsorption of the genomic DNA onto gold surfaces as a function of the level and distribution of methylcytosines, which is quantified by DPV in the presence of the $[Fe(CN)_6]^{3-/4-}$ redox system. Analysis of cell free DNA isolated from plasma samples of patients with different cancer types and from healthy individuals showed good discrimination; although, the detectability could be not enough to the early detection of the disease (need to distinguish as low as 1% methylation level). In this respect, it would be interesting to explore whether nanostructured gold surfaces could assist in addressing this issue without undermining the simplicity and rapidity of the methodology.

Another genomic alteration that appears in approximately 50% of PCa, while not found in BPH is the gene fusion between TMPRSS2 exon 1 and ERG exon 4 (TMPRSS2-ERG). This genomic rearrangement leads to a mRNA product that can be noninvasively detected in urine samples by quantitative polymerase chain reaction (Laxman et al., 2006). Trau's group developed two electrochemical approaches for the detection of this gene fusion product. One of them takes advantage of the superior adsorption affinity of adenine nucleobase onto bare gold surfaces. TMPRSS2-ERG mRNA partially hybridizes in solution with a biotin-tagged DNA probe. Then, it is captured onto streptavidin-modified magnetic particles, magnetically isolated, and released by heating at 95 °C for 2 min. The target is finally adsorbed through its polyA tail onto the gold transducer and quantified by DPV in the presence of ferri/ferrocyanide. A decrease in current intensity, inversely proportional to the mRNA adsorbed, is recorded. This amplification-free electrochemical genoassay was applied to detect the target in clinical urine specimens (Koo et al., 2016). As a step forward, integration of sample preparation (cell lysis), target amplification (solid-phase RPA) and chronoamperometric detection of an electroactive compound, whose generation is catalyzed by peroxidase-mimicking nanoenzymes serving as labels of the surface-confined amplicons, was carried out onto a biochip (Koo et al., 2018). This platform was used for interrogation of urine or 10-fold diluted serum clinical samples to detect not only TMPRSS2-ERG but also PCA3, SChLAP1, and KLK2 as internal control, thus turning out to be a convenient methodology for PCa liquid biopsies.

4. Perspectives and challenges for the future

Nucleic acid-based electrochemical sensors, reviewed in this work, offer a promising approach for the sensitive, rapid, and quantitative detection of PCa biomarkers. Advances in genomic and proteomic technologies have improved the understanding of the biology of prostate cancer and, at the same time, have led to the discovery of new biomarkers beyond PSA for its detection and monitoring. It is now clear that the development of PCa is due to multisystem and multilevel pathological changes. In consequence, clinical evidence based on a single biomarker seems not adequate for early diagnosis and the appropriate monitoring of its treatment. Electrochemical sensors using oligonucleotides as specific receptors are well suited for the detection of biomarkers at different molecular levels in samples as complex as urine or blood serum, offering excellent alternatives for the development of diagnostic devices useful for liquid biopsy. Outlining the key current challenges in this field might reveal priorities for future research.

The versatility of the biosensors herein described, which allows the detection of proteins, CTCs and exosomes or circulating nucleic acids, makes possible the screening of various analytes simultaneously. Genosensors and aptasensors have complementary applications and can be integrated into a single platform to provide data sets from a single sample, offering great potential to improve early detection and monitoring of response to therapy in prostate cancer. This multiplexing ability has already been explored (Wang et al., 2018; Yan et al., 2022), but it is in its infancy. The technical viability of multianalyte biosensors is well established and the assistance of microfluidics in their integration in POC devices suitable for clinical setting is actively investigated. Among all potential protein biomarkers, different combinations have been proposed (Aidoo-Brown et al., 2021), most of them including PSA, which is considered an indispensable tool in the management of PCa. However, a rigorous evaluation of the clinical validity is lacking. Therefore, the question about what biomarkers should be combined is still open and needs to be immediately addressed.

Evidences based on basic studies focused on finding the aberrant glycosylation that occurs in cancer cells, rather than in normal cells, suggest that this is a wide molecular field that is relatively unexplored, and may be the key for new cancer diagnostic and prognostic tools. The strategy of using aptamers for recognizing these cancer-associated modifications of glycans in PSA appears very promising to help in a more precise diagnosis of PCa (Díaz-Fernández et al., 2019, 2020).

An additional challenge is to demonstrate the value of CTCs as complementary tools in liquid biopsy. Cell-SELEX is raising aptamers that distinguish different types of PCa cell, e.g. metastatic or castration-resistant cells. Recently, a study of several aptamers against cancer cells including PCa showed low level of correlation with the target expression. This was attributed to the higher complexity of *in-vivo* systems when compared with cell cultures, thus indicating that a strong signal in cultures does not ensure successful translation to *in-vivo* activity. Interestingly, a robust aptamer has been selected for transferrin as a potential PCa biomarker (Kelly et al., 2021). The application of cell-derived aptamers is more and more focused on imaging and therapeutics, but their use in liquid biopsy should not be taken aside, especially when searching for exosomes or CTCs.

The detection of circulating nucleic acids as a complement to the

biomarkers mentioned above is also being explored. To date, miRNAs are the most studied. A great variety of electrochemical biosensing approaches have been proposed for PCa-related miRNA detection. Most of them exhibit good performance in buffered solutions with detectability within the range of attomolar to femtomolar. In many cases they have also demonstrated their ability to detect the target miRNA added to serum, plasma o urine conveniently diluted. However, for reliable quantitative analysis of these potential biomarkers, normalization to an endogenous reference gene is required to exclude any non-specific variation, thus demanding multianalyte sensing platforms. Likewise, standardization of sample preparation and RNA isolation are pending tasks.

Another relatively unexplored source of nucleic acid biomarkers are the lncRNAs. Apart from the urinary PCA3 test approved by US FDA, PCR-based tests have been commercialized for the detection of several nucleic acids in urine to enhance their clinical utility (Saini, 2016). This is the case of Mi-Prostate Score and Select MDx implemented after prostate massage. The first one performs the detection of PCA3 and TMPRSS2-ERG, demonstrating different clinical utility depending on the race (O'Malley et al., 2017). Select MDx detects two mRNAs, DLX1 and HOXC6, along with KLK3 for normalization. Exo Dx Prostate is another example of a commercially developed test that measures PCA3 and ERG in urinary exosomes without the need of a prostatic manipulation for obtaining the sample. All these tests, which have been commercially developed but not yet approved for their clinical use, can serve as inspiration for the design of genosensors against new lncRNAs explored for their clinical significance in the diagnosis of PCa.

There are clinical evidences suggesting that the biomarkers mentioned in this review can be used for the diagnostics, prognosis and prediction of the management of PCa patients. However, the different devices herein described are still in the research and development phase, and to move beyond the proof-of-concept step there are key issues that still need to be addressed: i) beyond the high sensitivity and specificity needed, which must be demonstrated in the biological samples (serum or urine), the stability and reproducibility of the sensing phase must be evaluated. Further studies are required to reduce batch to batch variations and enhance the stability of the devices exposed to complex sample media, for example, through biofouling in blood measurements; ii) ideally, devices should combine high-performance operation, minimal user intervention and low-cost fabrication. The use of advanced manufacturing technologies, for example 3D printing, in the manufacture of nucleic acid-based sensors could contribute to this; iii) standardization and quality control issues should be considered early, understanding the requirements of the industry and real-life end users for accelerating the translation to the clinical practice.

Finally, the use of computational tools of artificial intelligence (AI) is expected to improve the performance of multiplex platforms, accelerating their introduction into the clinical practice (Alcorcón-Zendejas et al., 2022). Therefore, this aspect demands increased attention. It is necessary to integrate electrochemical nucleic acid-based sensors and AI to develop advanced systems, which facilitates relevant clinical information for personalized management of PCa, thus improving the way this global public health problem in men is combated.

Despite the important advances in the development and analytical validation of the biosensors described in this review, there is still a long way to achieve their adoption in the clinical practice. For this, it is essential not only to demonstrate their ability to reliably and accurately measure the targets of interest, that is, to assess their clinical validity, but also to demonstrate their clinical utility. The later implies obtaining evidences of better clinical outcomes compared to the methods currently in use.

It is also possible to find a market niche for which there are no solutions available yet. The development of point of care devices for follow-up after prostate cancer treatment would be a good example. For this purpose, electrochemical devices are especially suitable. However, to achieve this goal, it is necessary to simplify the designs, making possible the direct measurement in a urine sample. Alternatively, microfluidic devices integrating all the analysis steps could be developed. In any case, the validation and subsequent regulatory completion of such a test is very laborious and time consuming, requiring a significant financial investment. Nevertheless, considering the ability of nucleic acid-based electrochemical sensors to be easily adapted to a point-of care format, we believe that this type of biosensors would drive numerous advances in the management of prostate cancer.

CRediT authorship contribution statement

Raquel Sánchez-Salcedo: Writing – review & editing. **Paula Gómez-Meijide:** Writing – review & editing. **Rebeca Miranda-Castro:** Conceptualization, Writing – review & editing, Supervision. **Noemí de-los-Santos-Álvarez:** Conceptualization, Writing – review & editing, Supervision, Funding acquisition. **María Jesús Lobo-Castañón:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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